



Selection and validation of appropriate reference genes for gene expression studies in *Forsythia*

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Abstract The qRT-PCR method has been widely used to detect gene expression level in plants, helping to understand the molecular mechanisms. However, there are few researches which focus on the selection of the internal reference genes in *Forsythia*. To select the appropriate reference genes of *Forsythia* aimed at qRT-PCR normalization, twelve candidate reference genes were selected from our transcriptome data. Their expression was assessed by RT-PCR analysis in 47 *Forsythia* samples, including 12 species cultivars, different organs and tissues. GeNorm, NormFinder, and BestKeeper software were used to select the appropriate reference genes, *AG* and *PSY* were used to verify the accuracy of the outcome. The results showed that *UKNI* was a stable reference gene in leaves of twelve *Forsythia* germplasms and in different developmental stages of fruits. *MTP*, *ABCT* + *MTP*, and *ABCT* + *MTP* + *TIP* were stable reference genes in different organs. *ACT* and *SDH* were stable reference genes in

different flower tissues and different developmental stages of the flower buds. When *Forsythia* plants were stressed with PEG or ABA, *SDH* + *UKNI* + *G6PD* was the stable reference gene group for qRT-PCR. The results provided the basis for investigating the physiological and biochemical processes of *Forsythia* related to medicinal and ornamental properties, and drought-resistance in the level of gene expression.

Keywords qRT-PCR · *Forsythia* · Reference genes · Flower · Fruit · Drought stress

Introduction

Forsythia belongs to *Oleaceae*, and is an important ornamental and medicinal plant (Ge et al. 2016; Rosati et al. 2007). The flowers of *Forsythia* are heterostylous, with the anther and stigma located at different heights (Long and Short) reciprocally (Sampson 1971). As an ornamental woody plant, *Forsythia* is widely found in the warm temperate zones of Asia and Europe, and plays an important role in the spring plant landscape (Rosati et al. 2007). The leaves and fruits of *Forsythia* are used most extensively for medical treatment and in functional diets (Guo et al. 2007; Piao et al. 2008). *Forsythia* is also listed as an herbal drug and food by the National Health Commission of the People's Republic of China (Ge et al. 2016). The main components of *Forsythia*'s leaves and fruit, which including lignans, forsythoside A–E, and flavonoids, have anti-inflammatory, anti-infective, anti-oxidant, and anti-viral pharmacological influential effects on the human body (Piao et al. 2008). These components are valuable and have broad prospects in clinical applications and drug development. In recent years, research on the functional aspects of

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Forsythia mainly focus on the medicinal components of different species and varieties, the anabolism of medicinal components, and the critical period for the harvesting of medicinal parts (Satake et al. 2013, 2015; Piao et al. 2008). As for the ornamental characteristics, previous studies mainly focused on the molecular mechanisms of flower color (Rosati et al. 2003), leaf color (Wang et al. 2017), cold resistance (Flinn and Ashworth 1999; Mcnamara and Pellett 1993), and drought resistance (Zhao et al. 2007). With advancements in technology, molecular techniques determining the level of gene expression have been introduced into the research of *Forsythia* (Satake et al. 2013, 2015; Shiraishi et al. 2016). Gene expression analysis is an important tool to advance these studies.

At present, the methods used in detecting plant gene expression include northern blot, in situ hybridization, RT-PCR, and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR is the most common method in current study, owing to its high sensitivity, specificity, accuracy, and high throughput (Gachon et al. 2004). Plant transcriptome sequence data and orthologous sequences of common housekeeping genes reported in model plant species were often used to screen for stable internal reference genes in non-model plants without the reference genome data (Li et al. 2017). However, the accuracy of the method is influenced by many factors, including plant materials, tissues and treatments, reverse transcription processes, and the amplification efficiency of primers. The optimal internal reference genes were varied among different species, different organs, and different developmental stages of specific species (Wang 2016; Hong 2016). Meanwhile, the use of improper reference genes for normalization in the relative quantification of gene expression could result in an incorrect conclusion (Gutierrez et al. 2010). Thus, for qRT-PCR analysis, it is important to screen for the optimal internal reference genes for different species and specific studies.

Appropriate reference gene selection has typically been reported for many plants used for herbal drugs and foods, such as *Lilium* (Xu et al. 2017), *Citrus* (Alves et al. 2018), *Amygdalus* (Kou et al. 2017). However, in *Forsythia*, genes selected from the *Forsythia* transcription data and housekeeping genes commonly used in model plant species were directly used as internal reference genes (Shiraishi et al. 2016). The application of these genes was limited in scientific research because they had not been standardized and tested systematically. There is still no report on the selection of an appropriate internal reference gene for *Forsythia*. In order to study the expression pattern and function of candidate genes in *Forsythia*, a selection of suitable internal reference genes is necessary. In our research, twelve candidate reference genes, often used as housekeeping genes or other stable reference genes in other plant species,

were selected from our transcriptome data and evaluated by geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), and BestKeeper (Pfaffl et al. 2004) algorithms in order to select appropriate reference genes for qRT-PCR normalization in *Forsythia*. The target genes agamous (*AG*) and phytoene synthase (*PSY*) were used as gene of estimate to test the stability of the selected reference genes, which were involved in flower development (Hou et al. 2010) and carotenoid synthesis (Domonkos et al. 2013) respectively. The selected internal reference genes include *UNK1*, which has the best function for some stages in other plants (Chi et al. 2012). Therefore, it is possible that *UNK1* performed as one of the stable reference gene in *Forsythia*. These selected internal reference genes could facilitate the study on gene expression during physiological and biochemical processes and play a significant role in the study of ornamental and medicinal molecular biology in *Forsythia*.

Materials and methods

Plant material

Forsythia seedlings were planted in July 2010 in the nursery of the National Engineering Research Center for Floriculture (NERCF) (40°02' N, 115°50' E), Beijing, China. The third fully expanded leaf of twelve *Forsythia* species and cultivars (Table 1) were collected on 20th July, 2018. Different plant organs of *F. intermedia* 'Courtaneur' were collected, including the root, stem, leaf, flower and fruit. During blossoming in the early spring of 2018, the flower buds of *F. intermedia* 'Courtaneur' were collected at different developmental stages and its fruits at different developmental stages were collected in autumn (Fig. 1). The development of the flower buds of *F. intermedia* 'Courtaneur' was divided into six stages, according to their shapes: grey bud (the buds are wrapped with grey flower scales) (Fig. 1a), green bud (the grey flower scales falls off, and the buds turn green) (Fig. 1b), start of yellow petals stage (half of the yellow petals are exposed) (Fig. 1c), fully yellow petals stage (the petals are fully exposed, but the flowers have not open) (Fig. 1d), start of blooming (the flowers start bloom) (Fig. 1e), and fully blooming (the flowers fully bloom) (Fig. 1f). Different parts of the mature flowers were then collected, (Fig. 1), including corolla (Fig. 1g), stamens (Fig. 1h), ovary and pistil (Fig. 1i), and sepals (Fig. 1j). The development of *F. intermedia* 'Courtaneur's fruits was divided into five stages: < 0.3 mm (Fig. 1k), 0.3–0.5 mm (Fig. 1l), 0.5–0.7 mm (Fig. 1m), 0.7–0.9 mm (Fig. 1n), and > 0.9 mm (Fig. 1o).

In June 2018, 1-year cutting plants of *F. intermedia* 'Courtaneur' were treated with abscisic acid (ABA)

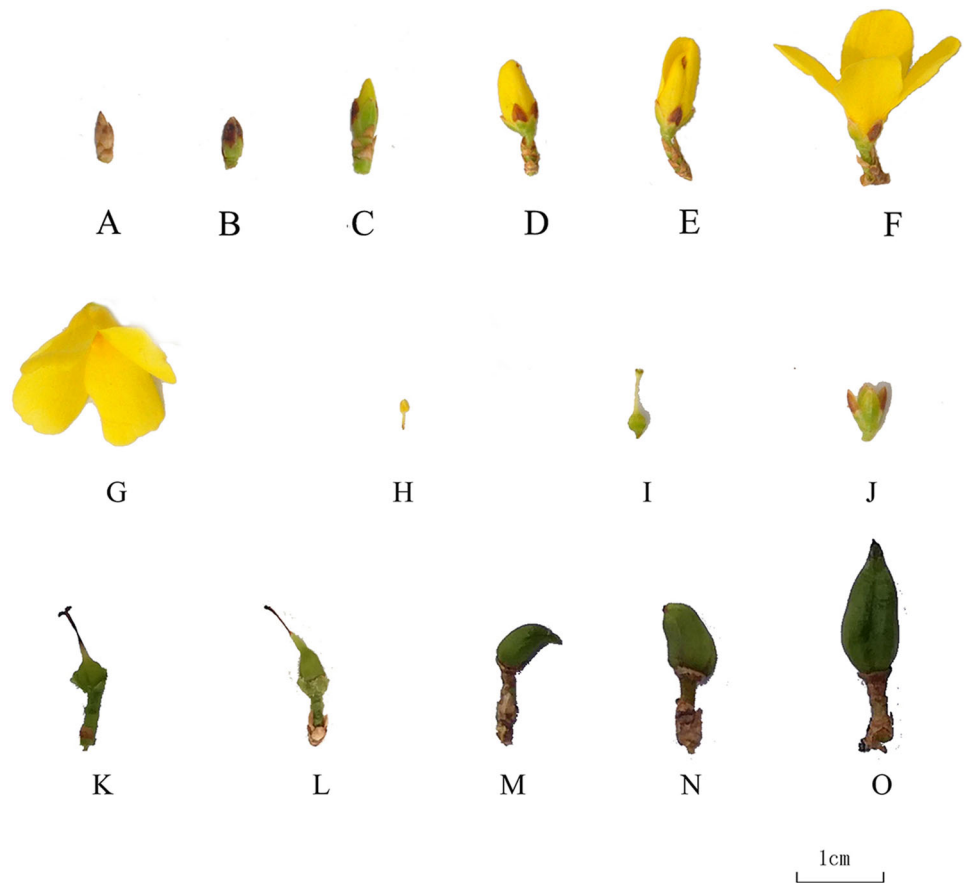
Table 1 Information of 13 *Forsythia* species and cultivars used in our research

Species	Cultivar	Style type	Code
Cultivars of <i>F. intermedia</i>	<i>F. intermedia</i> ‘Spectabilis’	S ^z	F1
	<i>F. intermedia</i> ‘Goldrausch’	S	F2
	<i>F. intermedia</i> ‘Lynwood’	S	F3
	<i>F. intermedia</i> ‘Courtalyn’	S	F4
	<i>F. intermedia</i> ‘Courtasol’	S	F6
	<i>F. intermedia</i> ‘Courtaneur’	L ^y	F7
	Cultivars of <i>F. koreana</i>	<i>F. koreana</i>	S
<i>F. koreana</i> ‘Sawon Gold’		S	FG
<i>F. viridissima</i> var. <i>koreana</i> ‘Kumson’		S	F8
Species	<i>F. mandshurica</i>	S	Fm
	<i>F. suspensa</i>	L	Fp
	<i>F. suspensa</i>	S	Ft
	<i>F. ovata</i>	–	Fo

^zS means stigma shorter than anther

^yL means stigma longer than anther

Fig. 1 The different developmental stages of flower buds and fruits, and different tissues of mature flowers of *F. ‘Courtaneur’*



(100 mg/L) and polyethylene glycol (PEG 6000) (50 g/L) for 48 h, respectively, simulating drought stress (Gong et al. 2018). The concentrations of ABA and PEG 6000 were selected through concentration gradient treatment experiment, the plants were treated with aqueous solution

containing different concentrations of ABA and PEG, then the semi-lethal concentration were sorted for further experiment. For each treatment fifty clones and their roots were soaked in the treatment solutions. After treatment, the plants were placed in the phytotron [temperature 25 °C,

light intensity $300 \mu\text{mol}/\text{m}^{-2} \text{ s}^{-1}$, 16 h/8 h (light/dark)] and the third pair of leaves were collected at 0 h, 2 h, 4 h, 6 h, 12 h, 24 h, 36 h and 48 h after treatment, respectively.

Three independent samples were taken at each stage or treatment.

Selection of candidate reference genes

Twelve candidate reference genes which have a stable expression ($-0.5 \leq \log_2 \text{ratio} \leq 0.5$) in the mature leaves of *Forsythia* and are often used as housekeeping genes in model plant species were selected from our transcriptome data (SRA accession number PRJNA437859, Supplemental data Table S1). According to their CDS sequences, the primers were designed on the Web using the Primer Quest Tool (<http://sg.idtdna.com/Primerquest/Home/Index>) and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), as shown in Table 2.

qRT-PCR analysis

The total cellular RNAs were extracted using the Trizol RNA reagent (Life Technologies, CA, USA) according to the manufacturer's instructions and were stored at -80°C . RNA was assessed using 1% agarose gel electrophoresis and was quantified with a UV-visible spectrophotometer (UV-2550, Japan). The RNA samples with A260/A230 ratios between 1.7 and 2.0 were used for subsequent analysis and were used to synthesize the first strand of cDNA with the Perfect Real Time RT reagent kit (Takara, Japan) in a 20 μl reaction mixture according to the manufacturer's protocol, once they were adjusted to the same concentration of RNA for each sample to 1 μg . The cDNA samples were diluted 1:5 with nuclease-free water prior to the qRT-PCR analysis.

qRT-PCR was performed using the PrimeScriptTM RT reagent kit with the gDNA Eraser (perfect real time) (Code No. RR047, Takara, Japan), and was restricted to the guidelines as follows: the Mini Opticon Real-time PCR (Bio-Rad Laboratories Inc., USA) reaction mixture (25 μl) contained 12.5 μl of SYBR Premix Ex Taq (TaKaRa Bio Inc., Japan), 2 μl of 1:5 diluted cDNA, 0.5 μl of each primer (10 mM), and 9.5 μl of water. The reactions were incubated under the following cycling conditions: 30 s at 94°C , 40 cycles of 94°C for 5 s, T_m 60°C for 30 s, and 72°C for 30 s, with a single melt cycle from 65 to 94°C in 5 s intervals.

The specificity of the primer pair was verified by the presence of a single peak in the melt curve analysis during the qRT-PCR process. Three independent biological replicates and three technical repetitions were performed for each of the quantitative PCR experiments. The threshold cycle (Ct) was measured automatically and the

correlation coefficients (R^2) together with the slope were calculated from the standard curve based on a fivefold series dilution of the cDNA templates. The corresponding qRT-PCR efficiencies (E) for each gene were determined from the given slope (Li et al. 2017).

Data analysis

GeNorm, NormFinder and BestKeeper algorithms were used to evaluate the stability of 12 candidate reference genes according to the Ct values, analyzing the stability of candidate reference genes comprehensively (Li et al. 2017).

Validation of reference genes

Agamous (*AG*) and phytoene synthase (*PSY*) were selected as control genes to confirm the reliability of the candidate reference genes, which are involved in flower development (Hou et al. 2010) and carotenoid synthesis (Domonkos et al. 2013). The stability in the expression of target genes *AG* and *PSY* was normalized using 5–8 genes that had been screened out. The qRT-PCR amplification conditions were the same as the conditions described above. *AG* was used to test the reliability of different developmental stages of flower buds and different parts of mature flowers, while *PSY* were used for other groups. The primer sequences of *AG* were as follows: Forward primer, 5'-TGAGCTCG-CAGCATCTTTAC-3', Reverse primer, 5'-GCTTGAC-CAGGATGTGGATATT-3'. The primer sequences of *PSY* were as follows: Forward primer, 5'-TCTCTCAGCAGACTCGTTATC-3', Reverse primer, 5'-CAGGGATGTTG GAGAAGAGTA-3'.

Results

Candidate reference genes and PCR amplification

Twelve candidate reference genes traditionally often used as housekeeping genes or stable reference genes in other plant species were identified on the basis of NR annotation, including genes of unknown function (*UKNI*), Cyclophilin (*CYP*), Beta Tubulin (*TUB*), Metalloprotease (*MTP*), Actin (*ACT*), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ATP-binding cassette transporter (*ABCT*), Succinate dehydrogenase (*SDH*), Tip₄₁-like protein (*TIP*), Polypyrimidine tract-binding (*PTB*), 6-phosphogluconic dehydrogenase (*G6PD*), and *F-Box*.

The amplification efficiencies of 12 candidate reference genes and 2 target genes ranged from 81.0 to 121.3%, and correlation coefficients ranged from 0.96 to 1 (Table 2). The single-peak melting curve showed that 14 primer pairs

Table 2 Reference genes and primers used for qRT-PCR in our research

Gene	Forward primer sequence	Reverse primer sequence	Amplicon length (bp)	Tm (°C)	Amplification efficiency (%)	Correlation coefficient (R ²)	Ct value
<i>UKNI</i>	CAGACCAGCTTTGAGGAGTATC	GGCCAGAAACCAGTAGTCAATA	92	60	98.0	0.976	21.00 ± 2.44
<i>CYP</i>	TGCCAGAAGGACCCTTAAATC	TGGAGGAAAGGCTTGTAAATC	83	60	115.5	0.989	23.46 ± 2.82
<i>TUB</i>	ACATTCCTGTGTCCCATC	CCATACACTCATCGCATCT	106	60	112.5	0.983	21.54 ± 2.32
<i>MTP</i>	GGTAGGCTGTCTCTTATCC	GGAAACCCATCCCGTACTT	110	60	102.3	0.992	21.81 ± 2.24
<i>ACT</i>	CGCCATCGAACTCCATAAA	TTGCAGAAGATGTCGGAACATA	90	60	81.0	0.960	19.64 ± 1.36
<i>GAPDH</i>	TGAAGGTGGTCTAAGAAAAG	GCAITGGAGACGATGTGTAGAT	115	60	98.0	0.982	20.98 ± 2.88
<i>ABCT</i>	GCTCCAGGACTACAGAGATTA	AGTTGAATGGGCACATATGG	95	60	121.3	0.995	20.51 ± 3.73
<i>SDH</i>	GAAATCGTCCCAACCCTTTACC	AGCTGTTCAAGGACAAGGTTATG	100	60	99.9	0.964	19.75 ± 2.21
<i>TIP</i>	TGCATACGAGTGTCCCTTAATC	CCCAGTGGTTGGTTTCTTCT	80	60	106.5	0.996	20.56 ± 2.10
<i>PTB</i>	GAACTCCCAGCCTTCCTTATT	GTATACTGGGCTCCGTTGTATG	84	60	104.0	0.996	21.73 ± 1.38
<i>G6PD</i>	TGCTCGGGCTTGATAGAAAC	GAAGGACGCGGTGGATATTT	119	60	91.2	0.989	21.28 ± 2.59
<i>F-Box</i>	GCCAGCCCAGTAGACATAAC	TTCCAAAAGTAAAGCCGACAAATC	100	60	95.1	0.982	22.39 ± 2.61

Ct (Cycle threshold) means the number of cycles experienced when the fluorescent signal in each reaction tube reaches the set domain value
Ct values are mean ± SE (n = 3)

generated single fragments, indicating non-specific products amplified in RT-PCR reactions (Fig. 2).

Expression stability of candidate reference genes

qRT-PCR analysis was used to detect the expression profiles of the 12 reference genes in different experimental materials, including the leaves of 12 *Forsythia* species and cultivars, different organs, different developmental stages of flower buds, tissues of flower parts, developmental stages of fruits, and the leaves of *Forsythia* plants treated with PEG and ABA. The results were shown in Fig. 3 and Table 2, which the mean CT values of the 12 reference genes ranged from 19.64 ± 1.36 (*ACT*) (mean ± SD) to 23.46 ± 2.82 (*CYP*). The expression of the 12 reference genes had a large fluctuation range and were not absolutely stable. Therefore, other calculation methods need to be introduced to compare the expression stability in different groups of samples, such as geNorm, NormFinder, and BestKeeper.

GeNorm analysis

The average expression stability value (M) of reference genes was evaluated using the geNorm program. The M value reflects the average variability between the different genes indicating that the lower the M value, the more stable the gene expression. However, if the M value is greater than 1.5, the gene is not suitable as a reference gene. The optimal number of reference genes was estimated by the coefficient of variation (V) between two genes, such as $V_n/V_{n+1} < 0.15$, indicating that no additional reference genes are needed to be added. Otherwise, the n + 1 gene are needed to be introduced (n is the number of reference genes) for accurate normalization (Vandesompele et al. 2002).

As shown in Fig. 4a, for the group of different *Forsythia* species and cultivars, the top 2 stable expression genes were *TIP* and *UKNI*, while *G6PD* need to be added for normalization owing to the value of $V_2/V_3 > 0.15$; *ABCT* was the least stable gene because of the value of $M > 1.5$. *ABCT* and *MTP* were the most stable reference genes among the different organs of *Forsythia*, while *TIP* was added for normalization owing to the value of $V_2/V_3 > 0.15$; *TUB*, *CYP*, *ACT*, *F-Box*, *PTB*, *GAPDH*, *G6PD* and *UKNI* were the least stable genes because of the value of $M > 1.5$ (Fig. 4b). *ACT* and *TIP* were the most stable reference genes in the different developmental stages of flower buds, while *SDH* was added for normalization owing to the value of $V_2/V_3 > 0.15$ (Fig. 4c). *SDH* and *UKNI* were the most stable reference genes in different tissue of flowers, while *ACT* was added for normalization owing to the value of $V_2/V_3 > 0.15$ (Fig. 4d). *G6PD* and

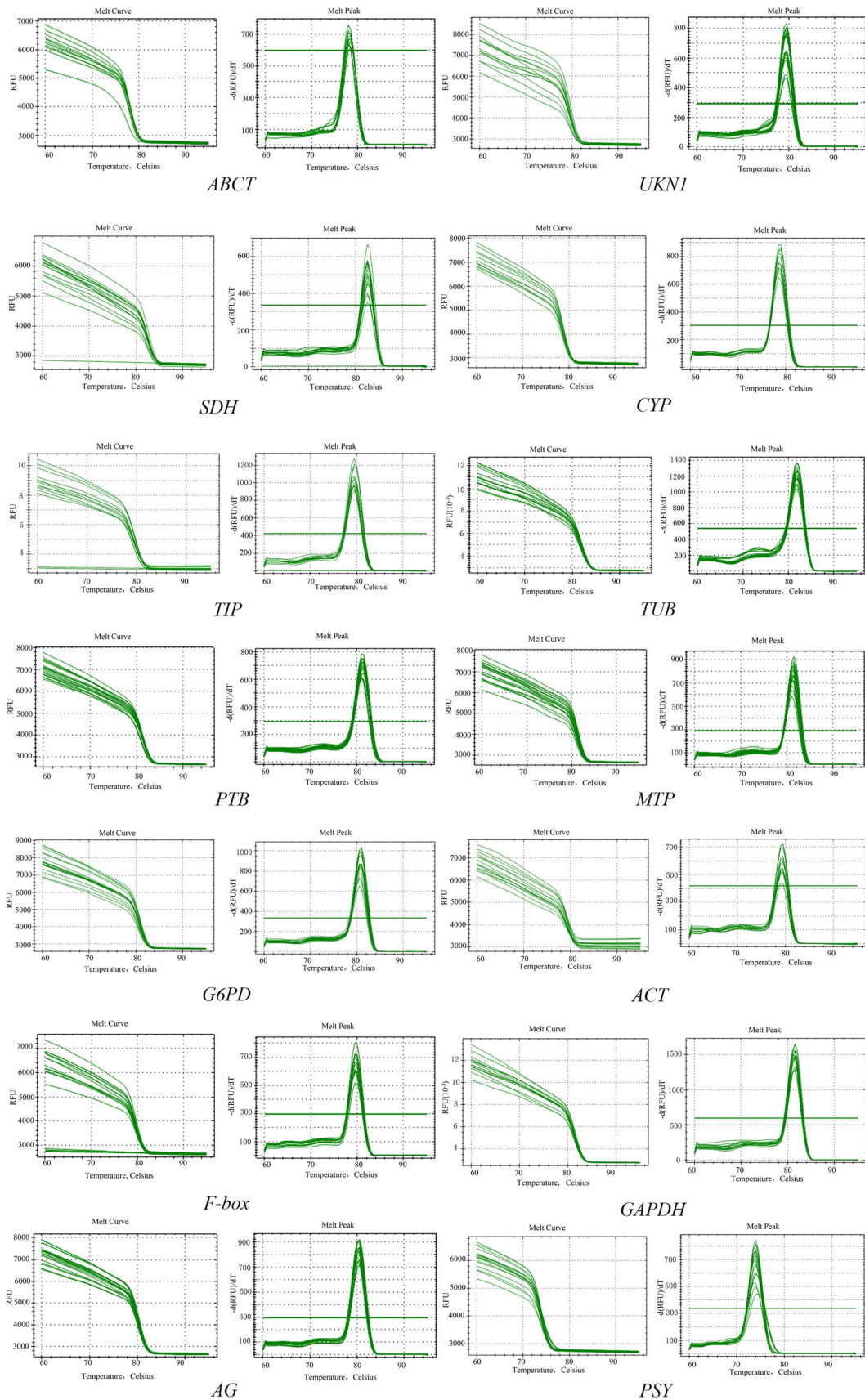


Fig. 2 Melting curves of the candidate reference genes and validate genes

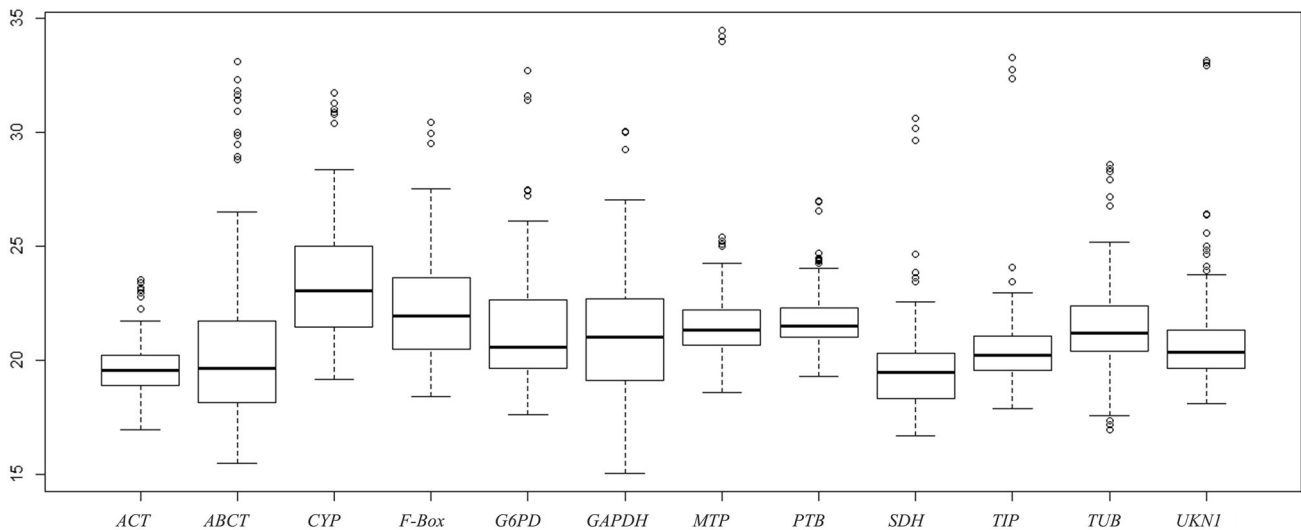


Fig. 3 Mean Ct values of 12 candidate reference genes. (Note: The data were pooled from all samples. The line across the box are the medians, the boxes depicts the 25/75 percentiles, the dotted lines represent the 95% confidence intervals and the dots are outliers)

UKNI were the most stable and suitable reference genes for normalization in different developmental stages of fruits, owing to the value of $V2/V3 = 0.15$ (Fig. 4e). *SDH* and *UKNI* were the most stable reference genes in mature leaves when plants were treated with ABA, while *ACT* was needed to be added for normalization owing to the value of $V2/V3 > 0.15$; *ABCT* and *GAPDH* were the least stable genes because of the value of $M > 1.5$ (Fig. 4f). *G6PD* and *SDH* were the most stable reference genes in mature leaves when plants were treated by PEG, while *UKNI* need to be added for normalization owing to the value of $V2/V3 > 0.15$, *ABCT* and *GAPDH* were the least stable genes because the value of $M > 1.5$ (Fig. 4g).

The results showed that the most stable reference genes varied in leaves of different *Forsythia* species and cultivars, as well as in different organs, different developmental stages of flower buds, different flower tissues, different developmental stages of fruits, and in mature leaves of *Forsythia* plants stressed with PEG and ABA.

NormFinder analysis

Further verification of the stability of reference gene was performed using NormFinder. The smaller the stability value, the more suitable it is as reference gene (Andersen et al. 2004).

Results are shown in Table 3, and the gene selected in different groups by geNorm and NormFinder show a similarity, including the leaves of different *Forsythia* species and cultivars (*TIP*, *UKNI* and *G6PD*), the different developmental stages of flower buds (*ACT*, *TIP* and *SDH*), and different part of flowers (*SDH*, *UKNI* and *ACT*), as well as mature leaves of plants treated with ABA (*SDH*, *UKNI*

and *ACT*). However, the selected reference genes using NormFinder in different developmental stages of fruits (*ACT*, *TIP* and *UKNI*) and mature leaves of plants treated with PEG (*G6PD*, *UKNI* and *SDH*) were different from the genes selected by the geNorm software. The most stable selected reference genes using NormFinder in different organs was *GAPDH*, which was quite different from the reference genes selected by the geNorm software.

BestKeeper

The stability of the reference genes was evaluated by the standard deviation (SD) and coefficient of variance (CV) of the Ct values in the BestKeeper program. The genes with an SD value below 1.0 were considered to be stable and could be used for the gene expression normalization. The smaller the value was, the more stable the gene was considered to be (Pfaffl et al. 2004). The program can only analyze the stability of 10 candidate reference genes at most, therefore, the top ten candidate reference genes for each group were selected for BestKeeper analysis according to the stability calculated by GeNorm and NormFinder.

The results are shown in Table 4. *UKNI*, *SDH*, *PTB*, *TIP*, *ACT*, and *G6PD* were selected as suitable reference genes in the leaves of different *Forsythia* species and cultivars. Only the *ACT* gene was selected as a suitable reference gene in different organs. Only *TIP* and *ACT* were selected as suitable reference genes in the developmental stages of flower buds. *ACT*, *PTB*, *TIP*, *MTP*, *ABCT* and *UKNI* were selected as suitable reference genes in different tissues of *Forsythia* flowers. *G6PD*, *UKNI*, *ACT*, *TIP*, *MTP*, *PTB*, *ABCT* and *SDH* were selected as suitable reference genes in different developmental stages of

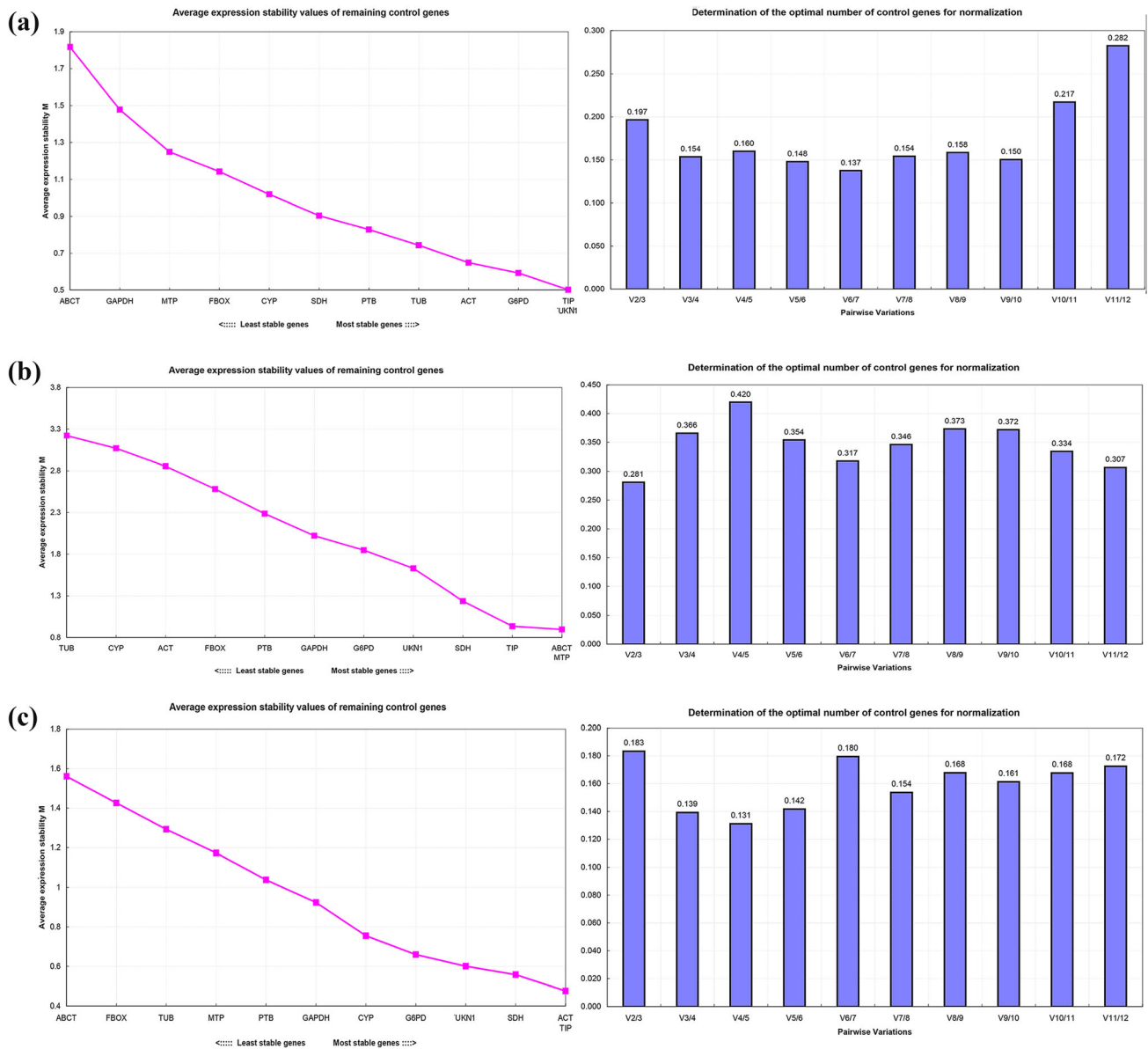


Fig. 4 Expression stability, ranking and pairwise variation of 12 candidate reference genes by geNorm software. (**a** different species and cultivars; **b** different organs of *Forsythia*; **c** different developmental stages of *Forsythia* flowers; **d** different tissue of *Forsythia*

lowers; **e** different developmental stages of *Forsythia* fruits; **f** leaves of *Forsythia* plants treated with ABA; **g** leaves of *Forsythia* plants treated with PEG)

Forsythia fruits. Only *TIP* and *MTP* genes were selected as suitable reference genes in mature leaves when *Forsythia* plants were treated by PEG. However, there was no suitable reference gene in mature leaves when *Forsythia* plants were treated by ABA for gene expression normalization.

In this study the stability of candidate reference genes was analyzed by the three software programs mentioned previously, and it was found that the most stable reference genes in the different developmental stages of the flower buds were *TIP*, *ACT* and *SDH*. The most stable reference genes in different developmental stages of the fruits were

ACT, *G6PD* and *UKNI*. The results among three analysis methods were consistent.

The most stable reference genes selected from different *Forsythia* species and cultivars, different tissues of flowers, and mature leaves of *Forsythia* plants treated with PEG and ABA were slightly different when they were analyzed with different software.

Validation of the reference genes

In order to examine the reliability of the candidate reference genes for normalization, the stable and

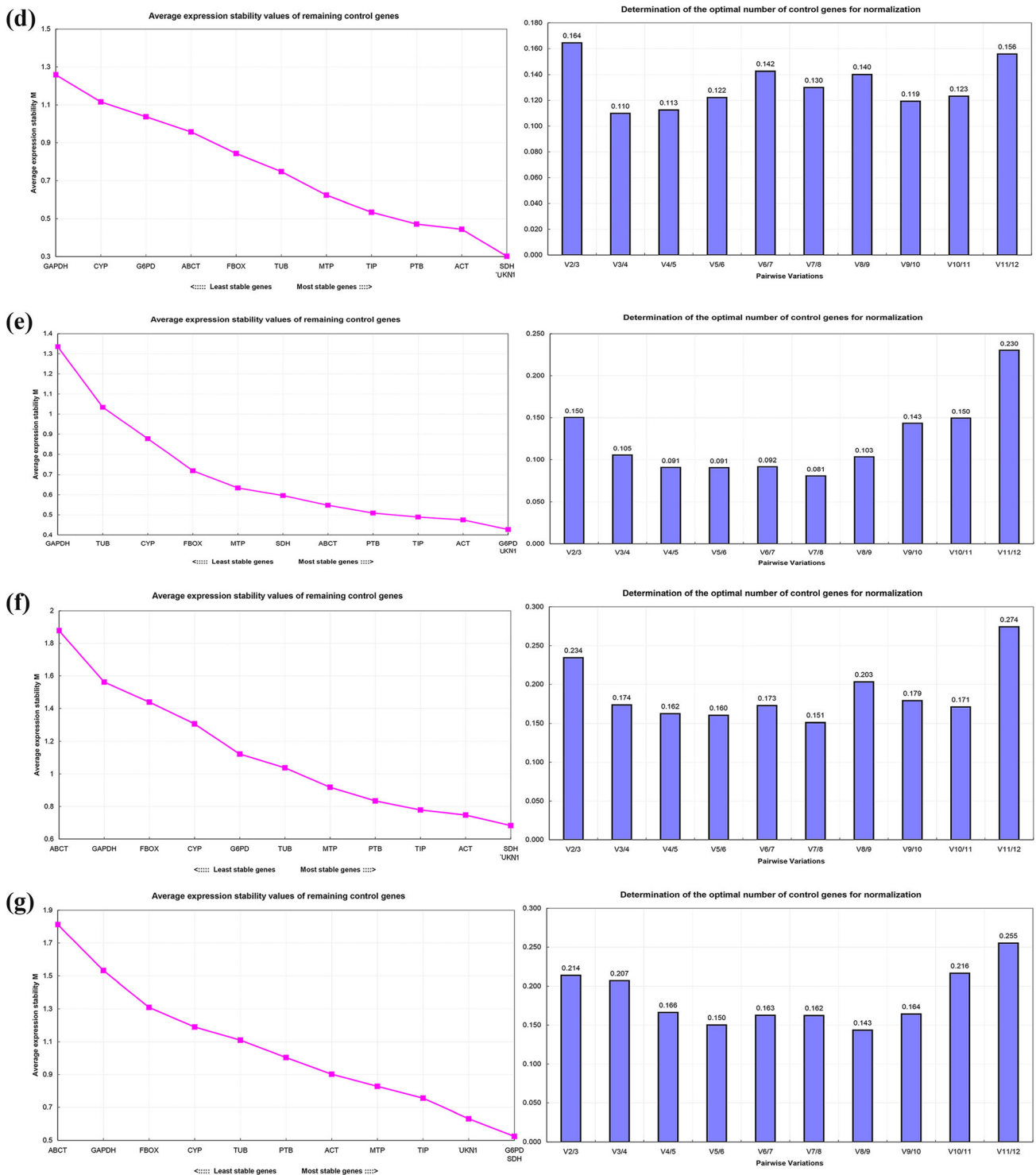


Fig. 4 continued

unstable reference genes recommended by the software analyses were used to normalize the expression trend of the test gene (*AG* or *PSY* gene) in different experimental materials. *AG* was chosen as the positive test gene in developmental stages and different tissues of *Forsythia*

flowers, while *PSY* was chosen as the positive test gene in the other experimental materials.

Candidate reference genes (*TIP*, *UKN1*, *G6PD*, *TIP* + *UKN1*, *TIP* + *UKN1* + *G6PD* and *MTP*) were used for normalization in the mature leaves of different *Forsythia*

Table 3 Ranking of the reference gene and their expression stability values calculated by Normfinder

Ranking order	Species and cultivars of <i>Forsythia</i>		Organs of <i>Forsythia</i> plants		Developmental stages of <i>Forsythia</i> flower buds		<i>Forsythia</i> flower plants		Developmental stages of <i>Forsythia</i> fruits		Leaves of <i>Forsythia</i> plants treated with ABA		Leaves of <i>Forsythia</i> plants treated with PEG	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	TIP	0.174	GAPDH	0.664	TIP	0.137	SDH	0.104	ACT	0.132	ACT	0.324	G6PD	0.036
2	UKN1	0.290	SDH	1.039	ACT	0.220	UKN1	0.139	TIP	0.140	TIP	0.491	UKN1	0.132
3	G6PD	0.323	G6PD	1.109	SDH	0.220	ACT	0.212	UKN1	0.143	PTB	0.533	SDH	0.182
4	ACT	0.410	PTB	1.182	G6PD	0.256	PTB	0.267	G6PD	0.148	UKN1	0.537	TIP	0.546
5	TUB	0.497	UKN1	1.357	UKN1	0.430	TIP	0.388	ABCT	0.263	G6PD	0.553	ACT	0.593
6	CYP	0.566	F-Box	1.488	CYP	0.561	TUB	0.524	PTB	0.341	SDH	0.591	MTP	0.647
7	SDH	0.626	TIP	1.803	GAPDH	0.818	MTP	0.575	MTP	0.397	TUB	0.665	TUB	0.700
8	PTB	0.724	ABCT	1.822	PTB	0.926	F-Box	0.641	SDH	0.432	MTP	0.912	CYP	0.792
9	F-Box	0.996	ACT	2.139	MTP	0.980	ABCT	0.901	F-Box	0.663	CYP	1.071	PTB	0.941
10	MTP	1.017	MTP	2.214	TUB	1.111	G6PD	0.912	TUB	1.071	F-Box	1.212	F-Box	1.096
11	GAPDH	1.732	CYP	2.252	F-Box	1.321	CYP	0.932	CYP	1.147	GAPDH	1.355	GAPDH	1.738
12	ABCT	2.337	TUB	2.503	ABCT	1.417	GAPDH	1.283	GAPDH	1.909	ABCT	2.268	ABCT	2.108

Stability values was calculated by Normfinder and the smaller the stability value, the more suitable it is as reference gene

Table 4 Ranking of the reference gene and their expression stability values calculated by Bestkeeper

Ranking order	Species and cultivars of <i>Forsythia</i>		Organs of <i>Forsythia</i> plants		Developmental stages of <i>Forsythia</i> flower buds		<i>Forsythia</i> flower plants		Developmental stages of <i>Forsythia</i> fruits		Leaves of <i>Forsythia</i> plants treated with ABA		Leaves of <i>Forsythia</i> plants treated with PEG	
	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability	Gene	Stability
1	UKN1	0.74	ACT	0.55	TIP	0.79	ACT	0.69	UKN1	0.18	MTP	1.08	PTB	0.95
2	SDH	0.75	PTB	1.48	ACT	0.98	PTB	0.71	G6PD	0.28	TIP	1.14	MTP	0.98
3	PTB	0.82	F-Box	1.86	SDH	1.05	TIP	0.72	ACT	0.34	PTB	1.28	TIP	1.15
4	TIP	0.82	SDH	3.15	G6PD	1.06	MTP	0.87	TIP	0.34	ACT	1.33	UKN1	1.42
5	ACT	0.87	GAPDH	3.24	PTB	1.07	ABCT	0.87	MTP	0.36	SDH	1.38	ACT	1.61
6	G6PD	0.96	TIP	3.83	UKN1	1.25	UKN1	0.91	PTB	0.39	UKN1	1.62	SDH	1.75
7	MTP	1.07	UKN1	3.94	CYP	1.26	SDH	1.06	ABCT	0.44	TUB	1.89	G6PD	1.94
8	TUB	1.15	ABCT	3.96	GAPDH	1.37	G6PD	1.49	SDH	0.59	G6PD	2.16	CYP	2.1
9	CYP	1.28	G6PD	4.13	MTP	1.52	TUB	1.55	F-Box	1.05	F-Box	2.74	TUB	2.21
10	F-Box	1.51	MTP	4.13	TUB	1.62	F-Box	1.67	CYP	1.22	CYP	2.87	F-Box	2.64

Stability values was calculated by Bestkeeper and the genes value below 1.0 were considered to be stable and could be used for the gene expression normalization

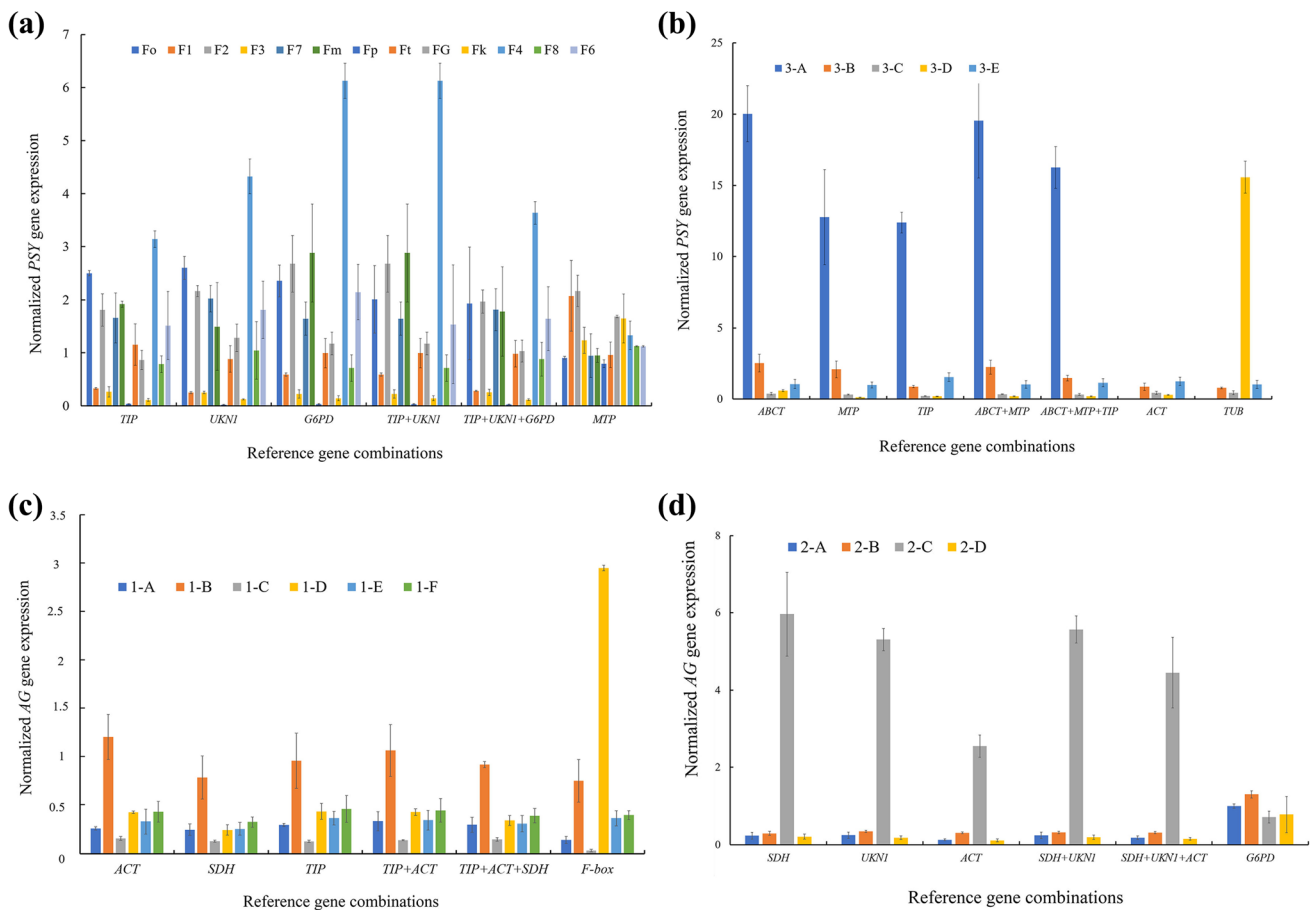


Fig. 5 Test of the stability of reference genes. **(a)** different *Forsythia* species and cultivars; **(b)** different organs of *Forsythia*; **(c)** different developmental stages of *Forsythia* flower buds; **(d)** different tissues of

Forsythia flowers; **(e)** different developmental stages of *Forsythia* fruits; **(f)** leaves of *Forsythia* plants treated with ABA; **(g)** leaves of *Forsythia* plants treated with PEG)

species and cultivars, only *UKNI* and *TIP + UKNI + G6PD* were suitable as the reference genes for qRT-PCR normalization through the *PSY* transcripts expression trend (Fig. 5A).

When *ABCT*, *MTP*, *TIP*, *ABCT + MTP*, *ABCT + MTP + TIP*, *ACT* and *TUB* were used for normalization in the different organs of *Forsythia*, only *MTP*, *ABCT+MTP* and *ABCT+MTP+TIP* were found suitable as the reference genes for normalization through the *PSY* transcripts expression trend (Fig. 5b). The most stable (*ACT*, *SDH*, *TIP*, *TIP + ACT*, *TIP + ACT + SDH*) and least stable (*F-box*) reference genes in different developmental stages of *Forsythia* flower buds were used for normalization, and the *AG* transcripts expression trend were all generally consistent except when *F-box* was used as the reference gene (Fig. 5c). The most stable (*SDH*, *UKNI*, *ACT*, *SDH + UKNI* and *SDH + UKNI + ACT*) and least stable (*G6PD*) reference genes in different tissues of *Forsythia* flowers were used for normalization, and the *AG* transcripts expression trend were all consistent except when *G6PD* was used as the reference gene (Fig. 5d). When *G6PD*,

UKNI, *G6PD + UKNI*, *ACT* and *MTP* were used for normalization in different developmental stages of *Forsythia* fruits, only *G6PD*, *UKNI* and *G6PD + UKNI* were found suitable as reference genes for normalization through the *PSY* transcripts expression trend (Fig. 5e).

SDH, *UKNI*, *G6PD*, *SDH + UKNI*, *SDH + UKNI + G6PD* and *CYP* were used for normalization in the mature leaves of *Forsythia* when plants were treated by ABA. However, only *SDH + UKNI* and *SDH + UKNI + G6PD* were suitable as reference genes for normalization through the *PSY* transcripts expression trend (Fig. 5f). *G6PD*, *UKV*, *SDH*, *G6PD+UKNI + SDH*, *ACT*, *TIP*, *PTB* and *CYP* were used for normalization in mature leaves when the plants were treated by PEG. Only *SDH* and *G6PD + UKV + SDH* were suitable as reference genes for normalization through the *PSY* transcripts expression trend (Fig. 5g).

In summary, the results suggested that the relative expression levels of the target gene are strongly affected by the choice of the reference genes.

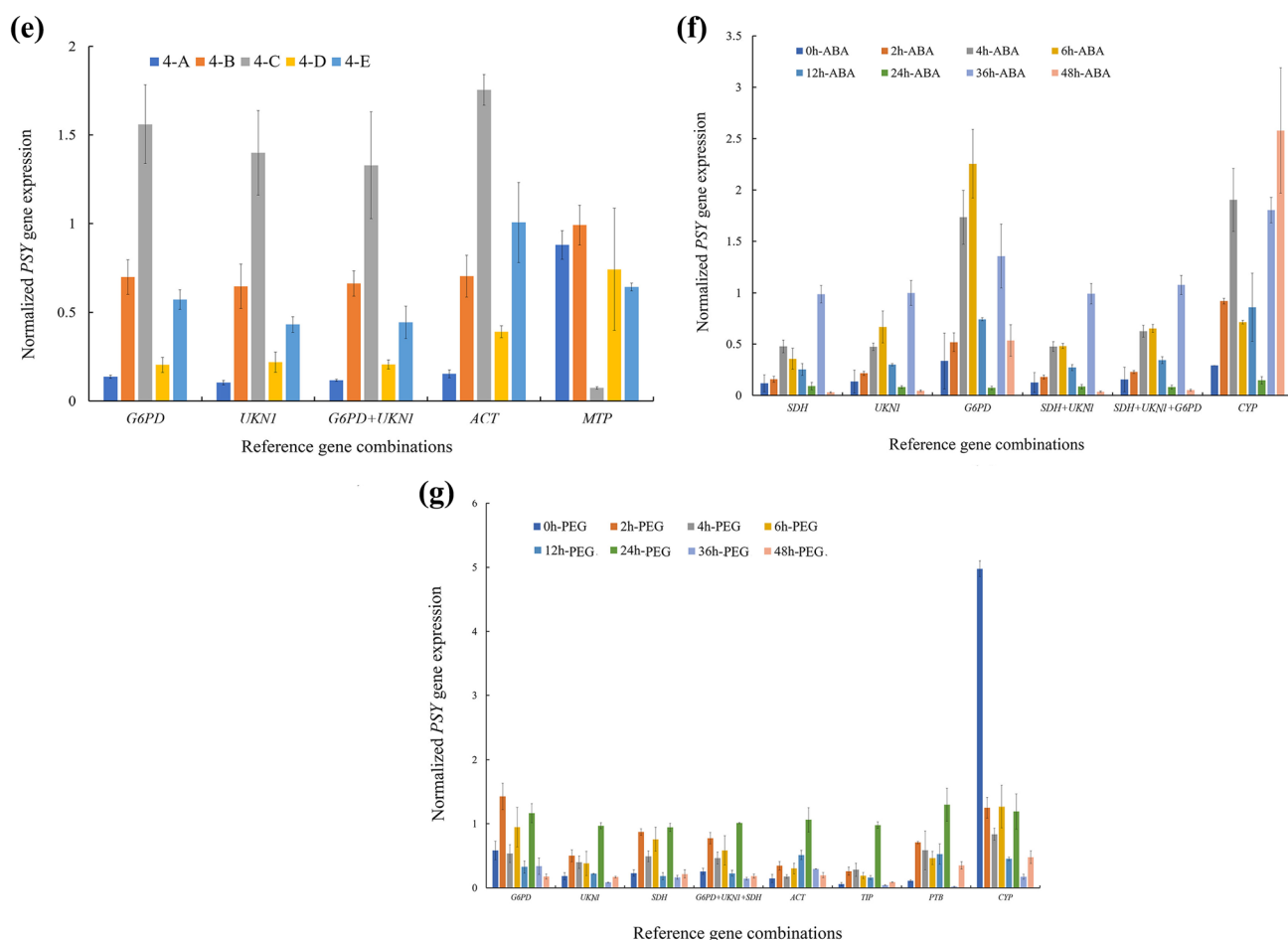


Fig. 5 continued

Discussion

Proper reference genes for normalization in qRT-PCR could provide insight into the complex regulatory networks in plant science research (Gutierrez et al. 2010). The optimal reference genes selected from different species, different development stages, and different organs of the specific species usually vary (Wang 2016; Hong 2016). *Forsythia* is an important ornamental and medicinal plant, however, there is no report on the identification and validation of a suitable reference gene for gene expression study in qRT-PCR (Guo et al. 2007; Piao et al. 2008; Flinn and Ashworth 1999; Mcnamara and Pellett 1993; Rosati et al. 2003; Wang et al. 2017; Zhao et al. 2007). In this study, the optimal reference genes for different *Forsythia* species and cultivars, different organs, different developmental stages, and different stress conditions were selected from 12 candidate genes, which provided a basis for studying the physiological and biochemical processes of *Forsythia* at the level of gene expression.

In order to select stable internal reference genes scientifically and accurately, diverse methods have been used to

evaluate the candidate reference genes, such as geNorm, NormFinder and BestKeeper (Wang 2016; Hong 2016). Due to different calculation methods, the stability of these internal reference gene candidates may be different. In our study the stability of candidate reference genes was analyzed by the three software programs mentioned before, and it was found that the most stable reference genes in the different developmental stages were consistent among three analysis methods, while these genes selected from different *Forsythia* species and cultivars, different tissues of flowers, and mature leaves of *Forsythia* plants treated with PEG and ABA were slightly different among three analysis method, and the results from geNorm and NormFinder analysis were more similar. However, the stable reference genes selected from different organs differed greatly, and the stability of the reference genes need to be further tested. Therefore, it is necessary to conduct comprehensive analyses in order to ensure that the selected internal reference genes could correctly normalize the results of qPR-PCR. In this study, the results of geNorm and NormFinder analysis were similar, but they showed differences when compared with the results obtained from

the BestKeeper analysis. For example, in the samples of different organs of *Forsythia*, *ACT*, *PTB* and *F-box* were selected by BestKeeper, and *MTP*, *ABCT* and *TIP* were selected by geNorm, and *GAPDH*, *SDH* and *G6PD* were selected by NormFinder. The stable reference genes selected by the three software analyses were examined by the *PSY* gene in different organs of *Forsythia* (Fig. 5b). We found that only *MTP* was suitable as internal reference gene, while *TUB*, *F-box*, *ABCT*, *TIP* and *ACT* were not suitable in different organs. And the same problem exists in citrus (Yan 2012). Therefore, it was necessary to use control genes to test the accuracy and validity of the selected reference genes. This study revealed that the results of the geNorm, NormFinder analysis were more accurate and reliable compared with the results of BestKeeper analysis.

In our study, the optimal internal reference genes selected from different species and cultivars, different organs, and different developmental stages of *Forsythia* varied (Table 5). *UKNI* was the stable reference gene in different species and cultivars of *Forsythia* and different developmental stages of *Forsythia* fruits, which was consistent with the most stable reference genes in soybean (Hu et al. 2009). *MTP*, *ABCT + MTP* and *ABCT + MTP + TIP* were the stable reference genes in different organs of *Forsythia*. *ACT* and *SDH* were the stable reference genes in different developmental stages and different tissues of *Forsythia* flowers. *SDH + UKNI + G6PD* was the stable reference gene combination when *Forsythia* was treated by PEG or ABA. While the stable internal reference genes of different organs, different developmental stages, and different stress treatment conditions were inconsistent with previous studies, such as those conducted with soybeans (Wang 2016), ash (Rivera-Vega et al. 2011), peach (Tong et al. 2009), and citrus (Yan 2012).

The results reveal that *UKNI* was the stable reference gene in the mature leaves of twelve *Forsythia* species and cultivars. Moreover, the expression stability of 12 candidate reference genes was assessed by the GeNorm, NormFinder, and BestKeeper software programs in different taxonomic groups of *Forsythia*, including in different species, different cultivars of *F. intermedia* and *F. koreana*, and different style types (Supplemental data Table S2, S3, S4 and Fig. S1). We found that *UKNI* was also the stable reference gene in the mature leaves of different taxonomic groups of *Forsythia*, but not the top ranked gene in these groups. *TIP*, *SDH*, *G6PD* were the most stable reference genes in different species and in different cultivars of *F. intermedia* and *F. koreana*. *TIP* was the most stable reference gene in the *Forsythia* species and cultivars with style type ‘S’ according to the result of software analysis, while stable reference gene recommended by the GeNorm, NormFinder and BestKeeper

software programs in *Forsythia* species and cultivars with style type ‘L’ had a large discrepancy when calculated by different software.

As mentioned above, it is extremely important to select the appropriate internal reference genes for the specific species and under specific experimental conditions. Selection of the optimal internal reference gene in *Forsythia* involved a molecular mechanism with consideration for the genetic expression of the medicinal and ornamental traits desired.

In our study, it was also found that *ABCT* and *TIP* were not suitable as internal reference genes for qRT-PCR normalization in different organs of *Forsythia*. However, *ABCT + MTP* and *ABCT + MTP + TIP* were suitable as internal reference genes for qRT-PCR normalization. It can be seen that the normalization, with a combination of multiple reference genes, could provide more accurate quantification of the relative expression than with a single gene in qRT-PCR analysis (Peng et al. 2006).

In summary, 12 candidate reference genes were selected based on our transcriptome sequence data and previous studies and their expression stability was assessed and selected in different species and cultivars, organs and tissues of *Forsythia*. This study could provide a basis for investigating the physiological and biochemical processes of *Forsythia* related to growth and development, drought-resistance, ornamental characteristics, and medicinal properties at the level of gene expression.

Conclusion

In our study, we selected appropriate reference genes in different species, cultivars, organs, tissues and stress conditions of *Forsythia* for the first time, filling the gaps for suitable reference genes referenced during the study of the regulatory mechanisms of *Forsythia* on metabolite biosynthesis, biological processes, and molecular breeding. Through systematic analysis and research (Table 5 and Fig. 5), we found that *UKNI* was the stable reference gene in mature leaves of twelve *Forsythia* species and cultivars and was also the stable reference gene in the developmental stages of the fruits. *MTP*, *ABCT + MTP* and *ABCT + MTP + TIP* were the stable reference genes in different *Forsythia* organs. *ACT* and *SDH* were the stable reference genes in different *Forsythia* flower tissues and developmental stages of the flower buds. *SDH + UKNI + G6PD* was the stable reference gene combination in the leaves of *Forsythia* when plants were treated by PEG or ABA. Our study may provide a basis for investigating physiological and biochemical processes of *Forsythia* in order to obtain more accurate and reliable data for the levels of relative gene expression.

Table 5 Expression stability ranking of the 12 candidate reference genes as calculated by software

Materials	Group	Software Ranking (stability of reference gene) (← most stable genes →) least stable genes →)													
		1	2	3	4	5	6	7	8	9	10	11	12		
<i>Forsythia</i>	12 species and cultivars	G*	UKV1	TIP	G6PD	ACT	TUB	PTB	SDH	SDH	CYP	FBOX	MTP	GAPDH	ABCT
		N*	TIP	UKV1	G6PD	ACT	TUB	CYP	SDH	SDH	PTB	FBOX	MTP	GAPDH	ABCT
		B*	UKV1	SDH	TIP	PTB	ACT	G6PD	MTP	TUB	TUB	CYP	FBOX	/	/
F. 'Courtaneur'	Different organs	G*	MTP	ABCT	TIP	SDH	UKV1	G6PD	GAPDH	PTB	FBOX	ACT	MTP	CYP	TUB
		N*	GAPDH	SDH	G6PD	PTB	UKV1	FBOX	TIP	TIP	ABCT	ACT	MTP	CYP	TUB
		B*	ACT	PTB	FBOX	SDH	GAPDH	TIP	UKV1	UKV1	ABCT	G6PD	MTP	/	/
Different developmental stages of flower buds	Different developmental stages of flower buds	G*	TIP	ACT	SDH	UKV1	G6PD	CYP	GAPDH	PTB	PTB	MTP	TUB	FBOX	ABCT
		N*	TIP	ACT	SDH	G6PD	UKV1	CYP	GAPDH	PTB	PTB	MTP	TUB	FBOX	ABCT
		B*	TIP	ACT	SDH	G6PD	PTB	UKV1	CYP	GAPDH	PTB	MTP	TUB	/	/
Different tissue of flowers	Different tissue of flowers	G*	UKV1	SDH	ACT	PTB	TIP	MTP	TUB	TUB	FBOX	ABCT	G6PD	CYP	GAPDH
		N*	SDH	UKV1	ACT	PTB	TIP	TUB	MTP	MTP	FBOX	ABCT	G6PD	CYP	GAPDH
		B*	ACT	PTB	TIP	MTP	ABCT	VKV1	SDH	SDH	G6PD	TUB	FBOX	/	/
Different developmental stages of fruit	Different developmental stages of fruit	G*	UKV1	G6PD	ACT	TIP	PTB	ABCT	SDH	SDH	MTP	FBOX	CYP	TUB	GAPDH
		N*	ACT	TIP	UKV1	G6PD	ABCT	PTB	MTP	MTP	SDH	FBOX	TUB	CYP	GAPDH
		B*	UKV1	G6PD	ACT	TIP	MTP	PTB	ABCT	ABCT	SDH	CYP	FBOX	/	/
Stressed by ABA	Stressed by ABA	G*	UKV1	SDH	ACT	TIP	PTB	MTP	TUB	TUB	G6PD	CYP	FBOX	GAPDH	ABCT
		N*	ACT	TIP	UKV1	G6PD	ABCT	PTB	MTP	ABCT	SDH	FBOX	TUB	CYP	GAPDH
		B*	UKV1	SDH	ACT	TIP	PTB	MTP	PTB	TUB	G6PD	CYP	FBOX	GAPDH	ABCT
Stressed by PEG	Stressed by PEG	G*	MTP	TIP	PTB	ACT	SDH	UKV1	TUB	TUB	G6PD	FBOX	CYP	/	/
		N*	SDH	G6PD	UKV1	TIP	MTP	ACT	PTB	PTB	TUB	CYP	FBOX	GAPDH	ABCT
		B*	G6PD	UKV1	SDH	TIP	ACT	MTP	TUB	TUB	CYP	PTB	FBOX	GAPDH	ABCT

G* means the gene stability calculated by GeNorm; N* means the gene stability calculated by NormFinder; B* means the gene stability calculated by BestKeeper

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